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Determining the Toxicity of UV Radiation and Chemicals on Primary and Immortalized Human Corneal Epithelial Cells

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TITLE:

Determining the Toxicity of UV Radiation and Chemicals on Primary and Immortalized Human Corneal Epithelial Cells

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primary culture, immortalized culture confocal microscopy, human corneal epithelial cells, calcein, ethidium homodimer, annexin V

SUMMARY:

This article describes the procedures used to evaluate the toxicity of UV radiation and chemical toxins on a primary and immortalized cell line.

ABSTRACT:

This article describes the methods of measuring the toxicity of ultraviolet (UV) radiation and ocular toxins on primary (pHCEC) and immortalized (iHCEC) human corneal epithelial cell cultures. Cells were exposed to UV radiation and toxic doses of benzalkonium chloride (BAK), hydrogen peroxide (H_2O_2), and sodium dodecyl sulfate (SDS). Metabolic activity was measured using a metabolic assay. The release of inflammatory cytokines was measured using a multi-plex interleukin (IL)-1β, IL-6, IL-8, and tumor necrosis factor-alpha (TNF-α) assay, and cells were evaluated for viability using fluorescent dyes.

The damaging effects of UV on cell metabolic activity and cytokine release occurred at 5 min of UV exposure for iHCEC and 20 min for pHCEC. Similar percent drops in metabolic activity of the iHCEC and pHCEC occurred after exposure to BAK, H_2O_2, or SDS, and the most significant

changes in cytokine release occurred for IL-6 and IL-8. Microscopy of fluorescently stained iHCEC and pHCEC BAK-exposed cells showed cell death at 0.005% BAK exposure, although the degree of ethidium staining was greater in the iHCECs than pHCECs. Utilizing multiple methods of assessing toxic effects using microscopy, assessments of metabolic activity, and cytokine production, the toxicity of UV radiation and chemical toxins could be determined for both primary and immortalized cell lines.

INTRODUCTION:

In vitro toxicology studies are performed to predict the toxic effects of chemicals and other agents that can cause damage to cells. In the assessment of toxicity to the cornea, human corneal epithelial cells (HCECs) have been used in models for evaluating these effects[1-4]. These models typically evaluate physiological effects such as changes to the cell's metabolic activity, cell proliferation, and other cell functions such as the production and release of inflammatory cytokines. For these toxicology studies, cells from various sources have been selected to assess the damaging effects of chemicals and UV radiation on HCECs[2,3]. pHCEC lines are available from companies that provide these cells from donor tissues of adults. Primary cells can be treated with dispase and gently scraped off the cornea for culture[5]. The cells are then tested for viruses and contamination and then shipped cryopreserved in 10% dimethyl sulfoxide.

The advantage of primary cell lines is that the cells are genetically identical to the cells of the donor. This is ideal, as an *in vitro* model should mimic the *in vivo* tissue as much as possible. The disadvantage of primary cell lines is that they have a limited number of cell divisions or passages[6]. The limited number of cells available restricts the number of experiments that can be conducted with a single primary culture, increasing the cost of the experiments.

Immortalized cell lines have also been used in cell culture toxicity models. However, unlike the primary cell line obtained from *in vivo* tissue, the immortalized cell line has been genetically altered. Immortalized cells are created by incorporating the genes of a virus into the DNA of primary cells[6-8]. Cells with successful viral gene incorporation are selected for the immortalized cell line. The advantage of immortalization is that it allows for indefinite rapid proliferation, providing an unlimited number of cells to perform multiple experiments using the same cell line. This allows for consistency between experiments and reduces the cost.

In addition to changes in the genes that limited cell proliferation, changes in the expression of genes of critical functionality could also occur[9]. Therefore, the disadvantage of using immortalized cells is that they may no longer represent the original *in vivo* cells in terms of their response to various external stimuli[10]. Comparisons have involved observing the toxic effects of chemicals on primary and immortalized human corneal keratocytes[11], as well as immortalized HCECs and rabbit corneal epithelial primary cells[12]. The comparison between the effects of toxins on primary human keratocytes and immortalized keratocytes showed no significant differences[11]. Using the methods detailed in this article, the effectiveness of these assays to assess the toxicity of UV radiation and ocular toxins on pHCECs and iHCECs will be determined.

Three ocular toxins commonly used in *in vitro* assays were selected: BAK, H_2O_2, and SDS. BAK is

a cationic preservative commonly used in ophthalmic solutions[13,14], H_2O_2 is commonly used to disinfect contact lenses[15], and SDS is an anionic surfactant found in detergents and shampoos[14]. Similar to ocular toxins, UV radiation can also cause significant damage to HCECs[3]. In addition, overexposure to UV can cause an ocular condition known as photokeratitis characterized by symptoms of tearing, light sensitivity, and a feeling of grittiness[16].

There is an unlimited number of primary cell cultures that can be used and various immortalized cell lines that have been developed. Therefore, an investigation was undertaken to compare primary HCECs to an immortalized HCEC line to determine similarities and differences between models that incorporate these types of cells.

This investigation used microscopy to assess possible differences between pHCECs and iHCECs on cell physiological response to UV and toxins. The effects of UV radiation and chemicals on cell metabolic activity and inflammatory cytokine release for the two cell lines were also evaluated. The importance of determining the differences among the two cell lines is to understand the optimal use of these cell lines for evaluating: 1) the effect of UV radiation on cells, 2) the effects of toxins on cells, and 3) the resulting changes to metabolism, cell viability, and cell cytokine release for future studies.

PROTOCOL:

1. Culture of pHCECs and iHCECs

1.1. Grow the pHCECs and iHCECs in human ocular epithelial medium (HOEM) with the following supplements: 6 mM L-glutamine, 0.002% cell media supplement O (**Table of Materials**), 1.0 µM epinephrine, 0.4% cell media supplement P (**Table of Materials**), 5 µg/mL rh insulin, 5 µg/mL apo-transferrin, and 100 ng/mL hydrocortisone hemisuccinate in collagen-1 coated culture flasks (18 mL in a 75 cm^2 culture flask).

1.2. Change the medium in the flasks every 2–3 days by removing the medium after the cells grow to ~80% confluence. Add dissociation solution without phenol red and incubate the flask at 37 °C until the cells are fully detached. Neutralize the dissociation solution with DMEM/F12 with serum and then centrifuge the cells at 500 × *g*. Remove the supernatant medium and resuspend the cells in HOEM medium.

2. Determination of cell size using confocal microscopy

2.1. Seed both pHCECs and iHCECs onto collagen-coated Petri dishes with glass-bottom coverslips at a concentration of 1×10^5 with 1 mL of HOEM. Grow pHCECs for 24 h and iHCECs for 48 h in a 37 °C incubator with 5% CO_2.

2.2. After the incubation period, stain the cells with 500 µL of annexin staining buffer solution containing calcein (4 µM), ethidium homodimer-1 (8 µM), and annexin V (5 µL in 500 µL buffer–Alexa Fluor 647 conjugate) for 20 min at 37 °C. After staining the cells, adjust the microscope

for capturing the excitation/emission wavelengths of 488/515 nm for calcein-AM, 543/600 nm for ethidium homodimer-1, and 633/665 nm for annexin V using a confocal laser scanning microscope.

2.3. Obtain the images and take Z-stacks to assess cell size in three dimensions.

2.3.1. To acquire the two-dimensional (2D) and three-dimensional (3D) images, find the area to image with an apochromat 40x/1.2 water objective. With the Argon (488) (first scan), HeNe1 (543) (second scan, and HeNe2 (633) (third scan) lasers, scan in three separate channels by using the beam splitters, HFT 488/543/633, NFT 635 Vis, and NFT 545 LP560, LP505.

2.3.2. Use multi-track sequential scanning to reduce crosstalk.

NOTE: LP505 is used for channel 2 with the 488 laser to capture the emission of the calcein dye. LP560 is used in channel 3 with the 543 laser to capture the emission of the ethidium homodimer 1. NFT 635 is used with the 633 laser to capture the emission of the annexin V. The purpose of the HFT is to separate the excitation and emission light. NFT splits light by reflecting light < specified wavelength to a separate channel while letting light > a specified wavelength through to a second channel. LP filters block shorter wavelengths than the specified wavelength and let the longer wavelengths through.

2.3.3. To image in 3D, set the confocal to Z-stack and scan at least 20 frames.

3. Exposure of cells to UV radiation

3.1. Seed the cells at 5×10^4/mL of HOEM in each well of a 24-well collagen-1 coated culture plate and incubate at 37 °C with 5% CO_2 for 3 h. After the incubation period, reduce the volume of the medium in the wells to 300 µL. Next, expose the cells to UV radiation (both UVA at 6.48 W/m^2 and UVB at 1.82 W/m^2 as both UVA and UVB tubes are turned on in the incubator at the same time) in a 37 °C incubator for 5 and 20 min in separate experiments.

3.2. After the UV radiation, add 200 µL of fresh HOEM to each well and incubate for 20 h at 37 °C with 5% CO_2.

3.3. After incubation, collect the cell supernatant in each well and transfer it into sterile 2 mL polypropylene tubes. Freeze at -80 °C. To determine the cytokine levels released by the pHCECs and iHCECs after UV exposure, use a multiplex cytokine assay and follow the kit's instructions to quantify the following four cytokines: IL-6, IL-8, IL-1β, and TNF-α.

3.4. Add metabolic assay reagent to the cells in the wells.

3.5. Prepare 10% metabolic assay reagent in DMEM/F12. Replace the culture medium in each well with 1 mL of the 10% metabolic assay solution and incubate at 37 °C with 5% CO_2 for 4 h.

3.6. Measure the fluorescence of each solution using a fluorescent plate reader at 530/590 nm excitation/emission wavelengths.

4. Exposure of cells to chemical toxins

4.1. Seed cells at 5 × 10^4/mL of HOEM in each well of a 24-well collagen-1 coated culture plate and incubate at 37 °C with 5% CO_2 for 3 h. After the incubation period, remove the medium and expose the cells to 1 mL of chemical toxins (BAK 0.001%, H_2O_2 0.01%, and SDS 0.0025% in phosphate-buffered saline (PBS)) for 5 and 15 min.

4.2. After exposure to the chemical toxins, remove the toxins from the wells, rinse with 1 mL of PBS, and add 1 mL of HOEM to each well. After 20 h of incubation, perform a metabolic assay by replacing the medium with 1 mL of a 10% metabolic assay solution and incubating at 37 °C with 5% CO_2 for 4 h. Measure the fluorescence using a fluorescence multi-well plate reader at 530/590 nm excitation/emission wavelengths.

4.3. Transfer the cell supernatants from the wells following the 20 h incubation into separate sterile 2 mL polypropylene tubes and freeze at -80 °C. Quantify cytokines released by the pHCECs and iHCECs after exposure to the chemicals. Use the same multiplex platform used to assess the cytokines from the UV-treated cells. Use a multiplex cytokine assay following the kit's instructions to quantify the following four cytokines: IL-6, IL-8, IL-1β, and TNF-α.

5. Examination of cells exposed to various concentrations of BAK

5.1. Seed cells at 1 × 10^5/mL of HOEM in each well of a 24-well collagen-1 coated culture plate and incubate at 37 °C with 5% CO_2 for 24 h. After the incubation period, remove the medium and expose the cells to 1 mL of chemical toxins (BAK 0.001%, BAK 0.005%, and BAK 0.01% in PBS) for 5 min.

5.2. After exposure, remove the chemical toxins, rinse the wells with 1 mL of PBS, and add 1 mL of HOEM to each well. After 20 h of incubation, stain the cells with 500 µL of annexin staining buffer solution containing calcein (4 µM), ethidium homodimer-1 (8 µM), and annexin V (5 µL in 500 µL buffer–Alexa Fluor 647 conjugate) for 20 min at 37 °C. Adjust the microscope to measure intensities of annexin V, calcein AM, and ethidium-1 staining at excitation/emission wavelengths of 630/675 nm, 495/515 nm, and 528/617 nm, respectively. Image the cells using the fluorescence microscope

6. Data analysis

6.1. Carry out a normality test and a test for equal variances before performing an analysis of variance (ANOVA), Welch ANOVA, or Kruskal-Wallis test. Use the appropriate *post-hoc* test for comparing each group tested. Set $p < 0.05$.

REPRESENTATIVE RESULTS:

Cell size

The primary and immortalized HCECs were visualized with three fluorescent dyes, which reflect three different stages of cell viability. Live cells are green (calcein-AM), dead cells are red (ethidium homodimer-1), and apoptotic cells are yellow (annexin V-computer-adjusted color for better visualization of the fluorescence signal). Live cells contain esterases in the cell cytoplasm and convert calcein-AM to calcein. Dead cells have cell membranes that are permeable to ethidium homodimer-1. Apoptotic cells have staining at the cell membrane as phosphatidylserine is translocated to the outer membrane.

A comparison of cell size for the two types of HCECs after 24 and 48 h of growth was made using confocal microscopy, as shown in **Figure 1**. The iHCECs (**Figure 1A**) are smaller cells that range from 10 µm to 20 µm, and the pHCECs (**Figure 1B**) range in size from 20 µm to 50 µm after 24 h of growth. Similar differences in size range were observed for iHCECs (**Figure 1C**) and pHCECs (**Figure 1D**) after 48 h of growth. 3D images of the two types of HCECs were made using confocal microscopy (**Figure 2A** shows pHCECs; **Figure 2B** shows iHCECs).

< INSERT FIGURES 1, 2A, and 2B >

Effect of UV on primary and immortalized HCECs

The metabolic activity of both primary and immortalized HCECs after exposure to UV is displayed in **Figure 3**. The figure shows normalized means from test wells (quadruplicate wells, two separate experiments). Compared to the non-UV exposed cells, the metabolic activity of irradiated pHCECs was significantly reduced at 20 min of exposure. For iHCECs, the metabolic activity decreased for cells irradiated at both 5 and 20 min. Therefore, it took a longer exposure time to reduce the metabolic activity of the pHCECs than the iHCECs.

< INSERT FIGURE 3 >

The effect of UV exposure on the release of inflammatory cytokines from the HCECs is shown in **Figure 4**. The maximum cytokine release occurred at different times of UV exposure for the primary and immortalized HCECs. The maximum cytokine release by the iHCECs was at 5 min of UV exposure. The cells released significant levels ($p < 0.05$) of IL-1β, IL-6, and IL-8 compared to the non-UV-exposed cells. Moreover, no significant cytokine release occurred at 20 min of UV exposure for the iHCECs. However, the maximum cytokine release for pHCECs occurred at 20 min of UV exposure. All four cytokines (IL-1β, IL-6, IL-8, and TNF-α) were released at significant levels compared to the non-UV -exposed cells. In terms of total amounts of inflammatory cytokines released (pg/mL), the pHCECs released substantially more IL-1β, IL-8, and TNF- α than the iHCECs, whereas the iHCECs released more IL-6.

< INSERT FIG 4 >

Effects of chemical toxins on primary and immortalized HCECs

The percentage reduction in metabolic activity after exposure to the three ocular toxins was similar between the pHCECs and iHCECs, as shown in **Figure 5**. The figure shows normalized means from test wells (quadruplicate wells, two separate experiments).

< INSERT FIG 5>

The amounts of cytokines released by the pHCECs were greater than the amounts released by the iHCECs. The release of cytokine IL-6 was impacted the most by exposure to the three chemicals (BAK 0.001%, H_2O_2 0.01%, SDS 0.0025%, **Figure 6**). The figure shows means (pg/mL) from test wells (quadruplicate wells, two separate experiments). Both pHCECs and iHCECs showed a change in the release of IL-6 after exposure to all three chemicals. BAK caused a decrease in the release of IL-6 compared to the control for both primary and immortalized HCECs, whereas H_2O_2 caused an increase in the release of IL-6 from iHCECs and a decrease in the release from pHCECs.

< INSERT FIG 6 >

Effects of various concentrations of BAK on cell viability

The effects of the BAK concentrations 0.001%, 0.005%, and 0.01% for 5 min on iHCEC viability are shown in **Figure 7**. BAK showed little effect at 0.001% for both pHCECs and iHCECs. Both pHCECs and iHCECs were significantly damaged at 0.005% and 0.01% of BAK. At 0.005% and 0.01% of BAK, the degree of ethidium staining was greater in iHCECs than pHCECs.

FIGURE AND TABLE LEGENDS:

Figure 1: A comparison of cell size using confocal microscopy. iHCECs after 24 (**A**) and 48 (**C**) h of growth with cells ranging in size from 10 to 20 µm. pHCECs after 24 (**B**) and 48 (**D**) h of growth with cells ranging in size from 20 to 50 µm. 40x water objective. Scale bars = 10 and 20 µm (**A**); 25 and 50 µm (**B**); 10 and 20 µm (**C**); 50 µm (**D**). Abbreviations: iHCECs = immortalized human corneal epithelial cells; pHCECs = primary human corneal epithelial cells.

Figure 2: 3D confocal images. 3D confocal images of pHCECs after 48 h of growth (**A**) and iHCECs after 24 h (**B**). 40x water objective. Abbreviations: iHCECs = immortalized human corneal epithelial cells; pHCECs = primary human corneal epithelial cells.

Figure 3: The metabolic activity of pHCECs and iHCECs after exposure to 5 and 20 min of UV radiation. *$p < 0.05$ of the non-UV-exposed control. The non-UV-exposed control are cells in wells incubated with the test samples but not exposed to UV. Y-axis is percent relative to the metabolic activity of the cells not exposed to UV. Error bars indicate standard deviation. Abbreviations: iHCECs = immortalized human corneal epithelial cells; pHCECs = primary human corneal epithelial cells; UV = ultraviolet.

Figure 4: Cytokine release by the pHCECs and iHCECs after exposure to UV radiation in pg/mL. The four proinflammatory cytokines quantified are IL-1β (**A**), IL-6 (**B**), IL-8 (**C**), and TNF-α (**D**). *p < 0.05 of the non-UV-exposed control. Error bars indicate standard deviation. Abbreviations: iHCECs = immortalized human corneal epithelial cells; pHCECs = primary human corneal epithelial cells; UV = ultraviolet; IL = interleukin; TNF-α = tumor necrosis factor alpha.

Figure 5: The metabolic activity of pHCECs and iHCECs after exposure to three ocular toxins for 5 and 15 min. *p < 0.05 of the control. The non-toxin-exposed control are cells in wells incubated with the test samples but not exposed to chemical toxins. Y-axis is percent relative to the metabolic activity of the cells not exposed to chemical toxins. Error bars indicate standard deviation. Abbreviations: iHCECs = immortalized human corneal epithelial cells; pHCECs = primary human corneal epithelial cells; BAK = benzalkonium chloride; H_2O_2 = hydrogen peroxide; SDS = sodium dodecyl sulfate.

Figure 6: Cytokine release. Cytokine release by the pHCECs and iHCECs after exposure to three ocular toxins for 5 and 15 min in pg/mL. The four proinflammatory cytokines quantified are IL-1β (**A**), IL-6 (**B**), IL-8 (**C**), and TNF-α (**D**). *p < 0.05 of the control. Error bars indicate standard deviation. Abbreviations: iHCECs = immortalized human corneal epithelial cells; pHCECs = primary human corneal epithelial cells; BAK = benzalkonium chloride; H_2O_2 = hydrogen peroxide; SDS = sodium dodecyl sulfate; IL = interleukin; TNF-α = tumor necrosis factor alpha.

Figure 7: Fluorescence microscopic images of pHCECs and iHCECs exposed to different concentrations of BAK for 5 min. Scale bars = 200 μm; 10x objective. Abbreviations: iHCECs = immortalized human corneal epithelial cells; pHCECs = primary human corneal epithelial cells; BAK = benzalkonium chloride.

DISCUSSION:

Potential differences in the use of two types of HCECs were assessed. Cells were placed in the same medium (HOEM) at identical concentrations of cells and then exposed to short and long periods of UV radiation and three ocular toxins. Doses of UV radiation and chemicals were selected based on their physiological effects, which were damaging enough to the cells to produce intermediate responses that could be compared. Exposure times of 5 and 20 min for UV radiation and 5 and 15 min for the selected doses of BAK (0.001%), SDS (0.0025%), and H_2O_2 (0.01%) were ideal exposure times and concentrations that showed significantly different responses between the cells and the untreated controls.

HOEM is a serum-free medium that is optimized for the culture of HCECs. HOEM supports the growth of both primary and immortalized cells. The immortalized cell line used in this investigation was immortalized using SV40[8]. SV40-immortalized cells express oncogene proteins that can promote cell cycle progression by interfering with Rb, p53, and pp2a[17]. Retinoblastoma (Rb) binds to and inhibits E2F transcription factors, which, when not inhibited, allow a cell to progress in the cell cycle and divide[18]. The molecule p53 also binds to transcription factors to control the progression of the cell cycle[19]. The inhibition of a third molecule, pp2a[20,21], also

promotes cellular proliferation[22]. The expression of SV40 oncogene proteins that inhibit Rb, p53, and pp2a[17] may contribute to the differences in the response of iHCECs vs. pHCECs. The cell sizes of the two types of HCECs were also different, which may be due to these oncogene-inhibiting proteins because the immortalized cells are forced to divide by these molecules before the cells achieve the large size of the primary cells.

Corneal exposure to UV radiation can cause severe damage to corneal epithelial cells. UV radiation can produce reactive oxygen species in the cell, damage DNA and cell molecules, and disrupt enzyme processes[23,24]. UVB directly damages DNA, and UVA damages DNA and other cellular molecules via the production of reactive oxygen species within the cell that can then react with other biomolecules[25,26]. This UV damage can cause cytotoxicity and inflammation due to the release of inflammatory cytokines from the exposed cells[3,27]. In this study, immortalized cells were more sensitive to UV radiation effects on cell metabolic activity, as there was a drop in the metabolic activity of the immortalized cells but not in the primary cell line after 5 min. Differences in the release of inflammatory cytokines also occurred between the pHCECs and iHCECs. pHCECs released more IL-1β, IL-8, and TNF-α than iHCECs after 20 min of UV radiation. These differences in cytokine release could be related to the differences between the cell size of primary and immortalized cell cultures. Differential effects in cytokine release between primary and immortalized cells have been noted in another study that examined the effects of cigarette smoke on primary and immortalized cell lines[28].

To model the potential of chemical toxins to cause ocular irritation, various cell culture models have been developed to assess the cytotoxicity of these chemicals. This study showed that ocular toxins that have intermediate effects on HCECs have approximately the same percentage decrease in metabolic activity for both primary and immortalized HCECs. The three ocular toxins did not cause a substantial release of most of the inflammatory cytokines compared to the untreated control. However, this paper shows the sensitivity of this method in detecting baseline cytokine levels. Another method for detecting cytokines from immortalized cell lines has been evaluated and was found unable to detect baseline cytokine levels[29]. The microscopic images of BAK-exposed cultures showed toxicity in both pHCECs and iHCECs at the 0.005% and 0.01% concentrations after 5 min exposure and 20 h recovery.

The critical steps in this protocol were selecting UV doses and ocular toxin doses that resulted in intermediate toxicity to the cells and ensuring that each type of HCECs grew in the same medium. This allowed for comparisons to be made between the effects of each treatment on the pHCECs and iHCECs. Modifications of this method can be made in the time of exposure to the toxic agents. However, it is recommended to maintain exposure time close to the times tested in this protocol as shorter time points may not show toxicity on the cells. In contrast, too long an exposure may cause too much damage so that minor differences in the toxicity of ocular toxins could not be compared to the damaging effects of the test agents described in this protocol.

One of the advantages of the immortalized HCECs over the primary HCECs is that the immortalized cells had lower standard deviations than the primary cells for metabolic activity

and cytokine release after exposure to ocular toxins. Thus, for assessing the metabolic activity and cytokine release after exposure, the immortalized cells are more likely to detect physiological toxicity than primary cells. Both the primary cells and immortalized cell line detected significant effects on metabolic activity and cytokine release after UV exposure, and both detected the toxicity of BAK after staining the cells with fluorescence dyes.

In addition to the toxicity endpoints mentioned in this investigation, other endpoints could be tested, including effects on tight junctions, cell proliferation, cell migration, and the release of additional cytokines. This protocol evaluated the effects of toxic agents on the release of four inflammatory cytokines, metabolic activity, and cell viability using measurements for cell permeability, esterase activity, and apoptosis. In addition, *in viv*o tests, such as rabbit ocular irritation testing, should be included in a toxicity test battery, as i*n vitro* toxicity tests are suitable for assessing the mechanism of action of toxicity to HCECs. *In vivo* tests in animals are needed to determine if other immune and toxicity mechanisms exist that cannot be modeled *in vitro*.

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Figure 1

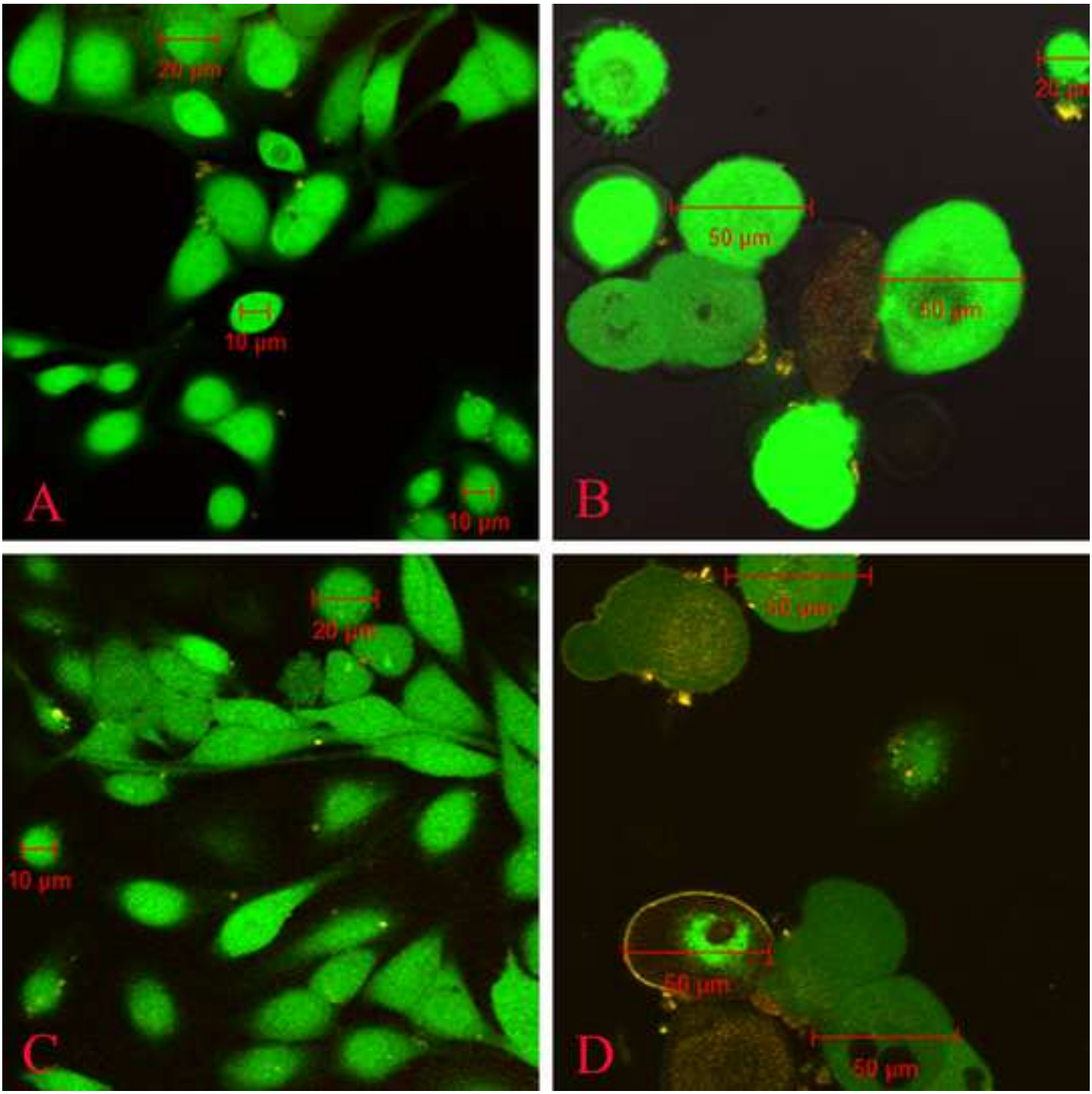

Figure 2

Click here to access/download;Figure;fig 2 with a and b.tif

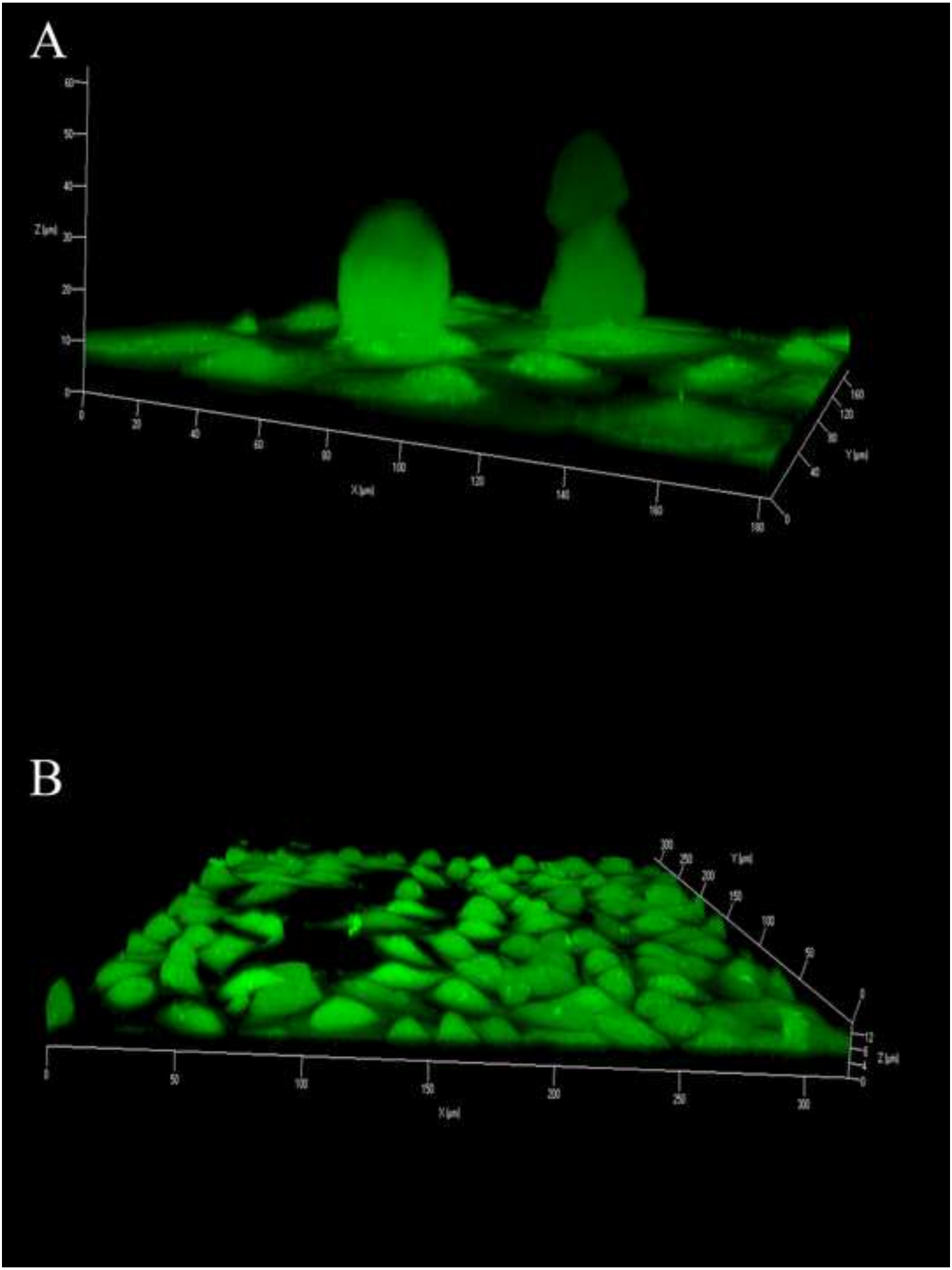

Figure 3

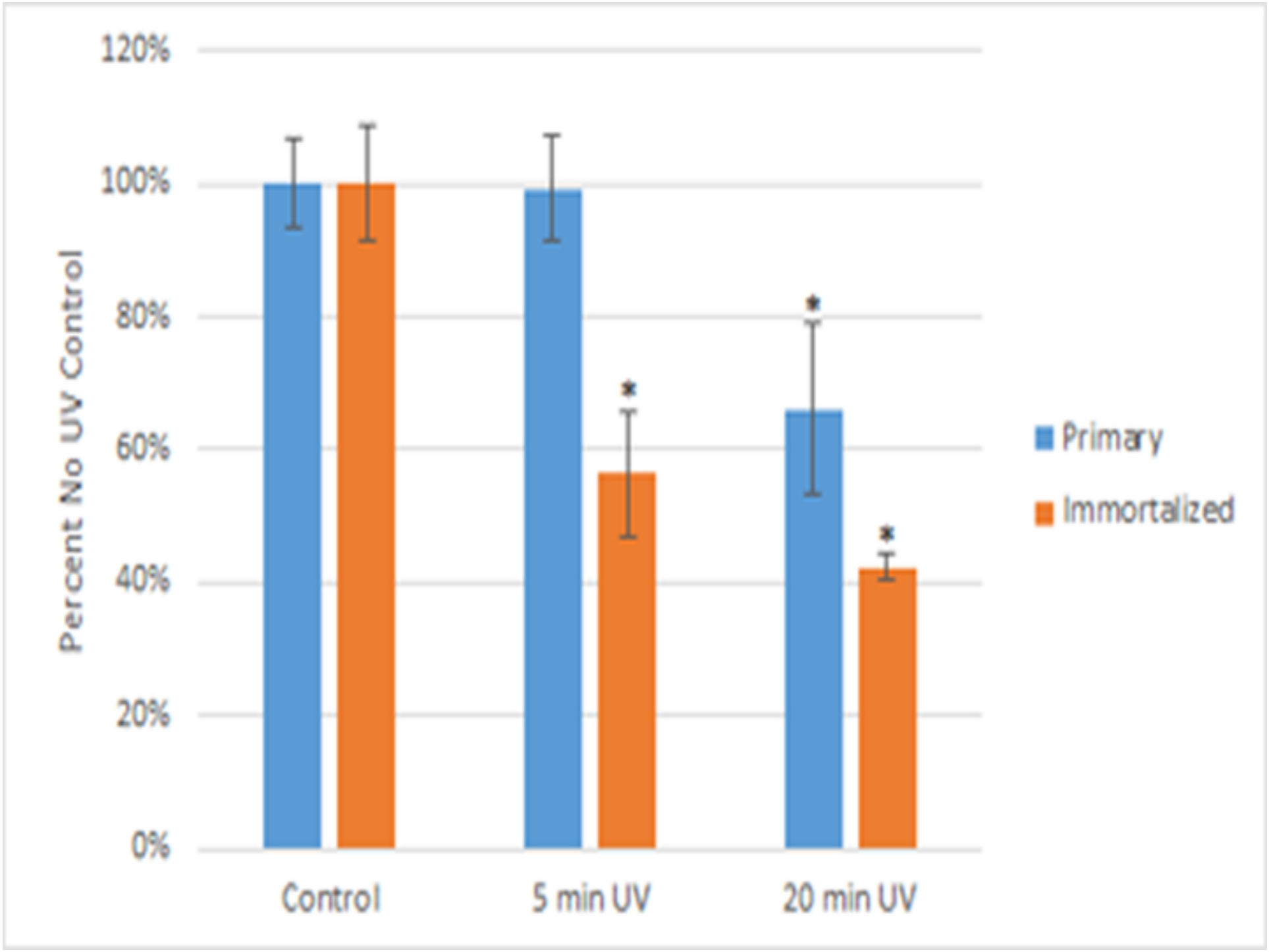

Figure 4

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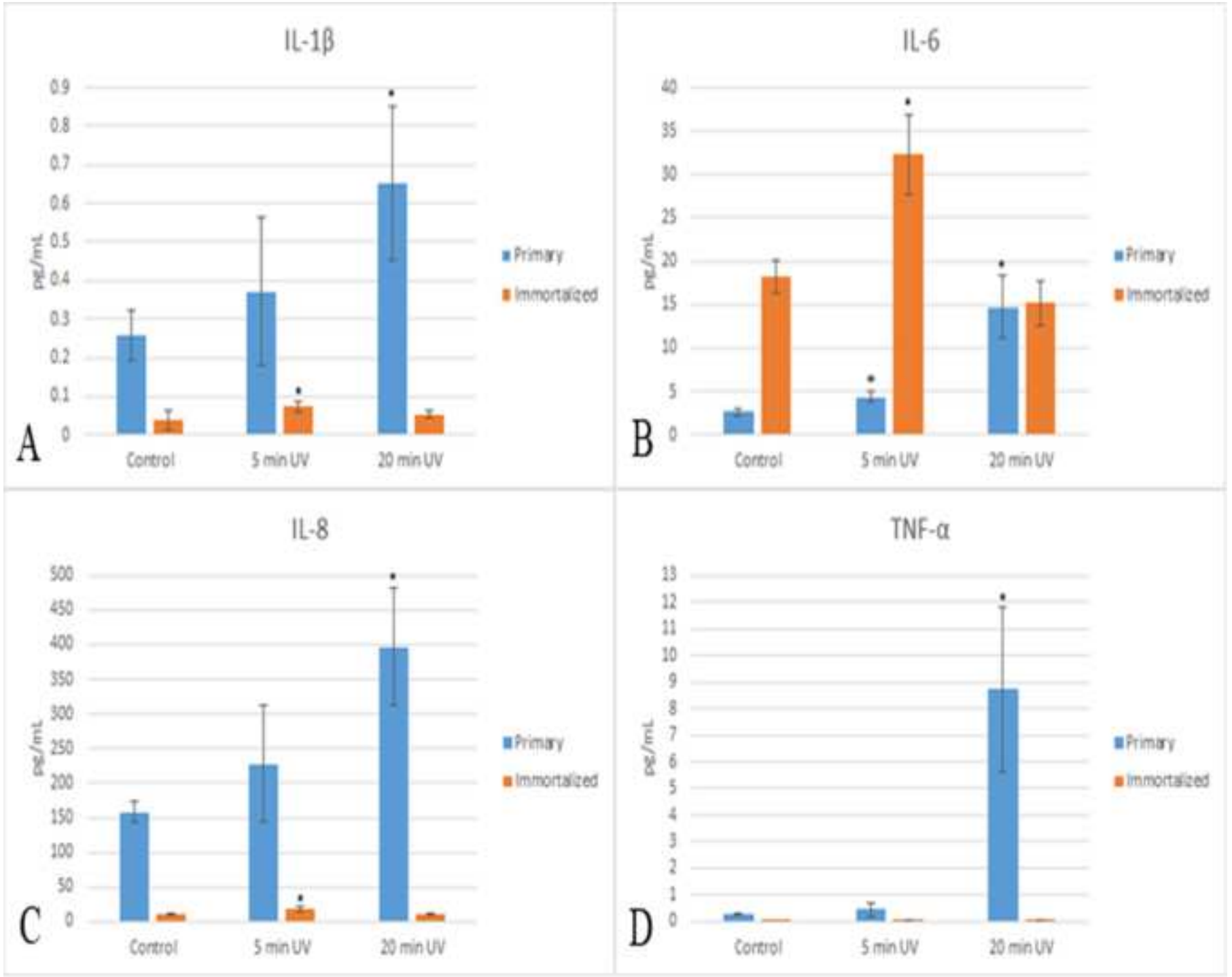


Figure 5

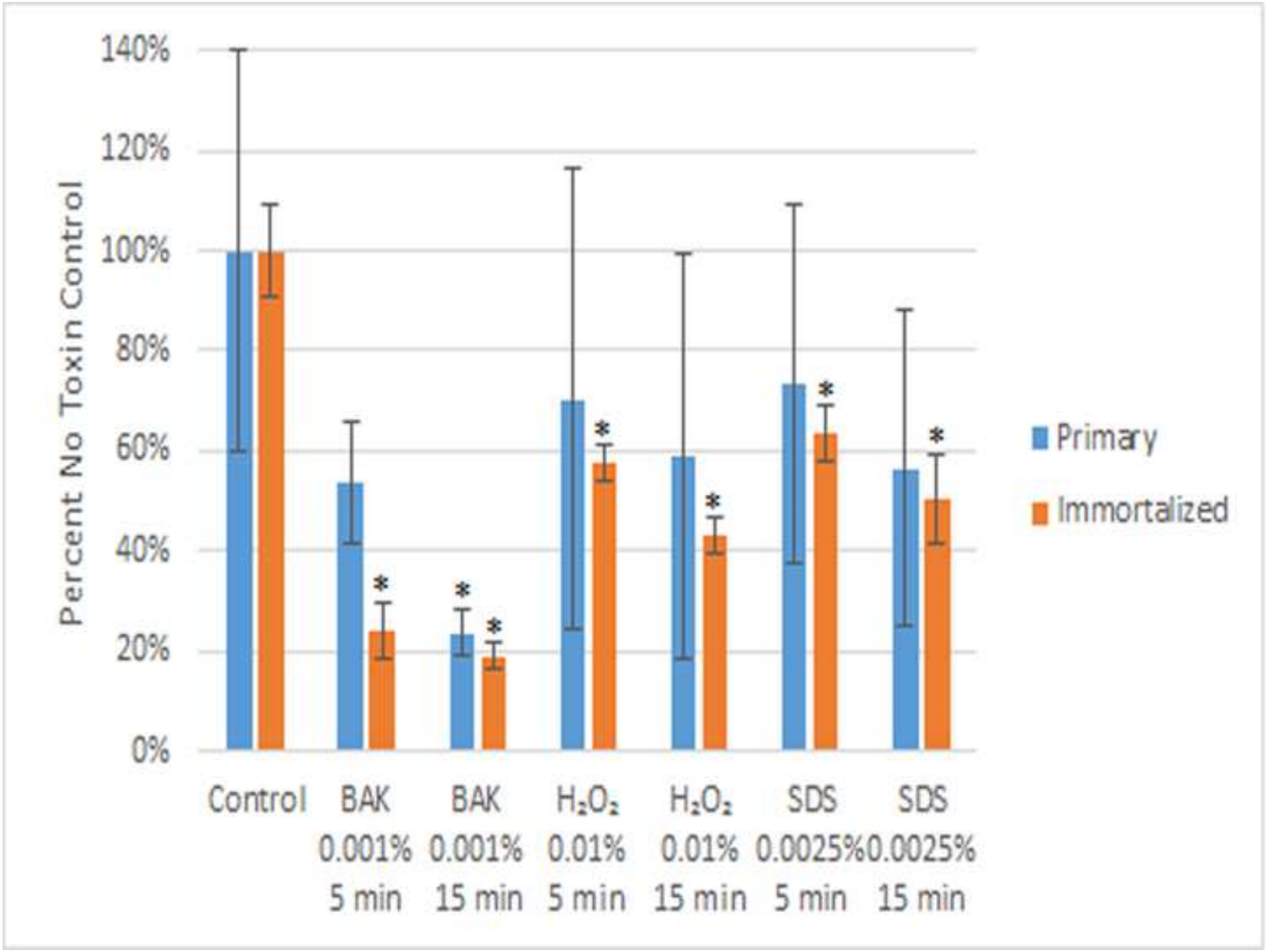


Figure 6

Click here to access/download;Figure;Figure 6-adjusted.tif

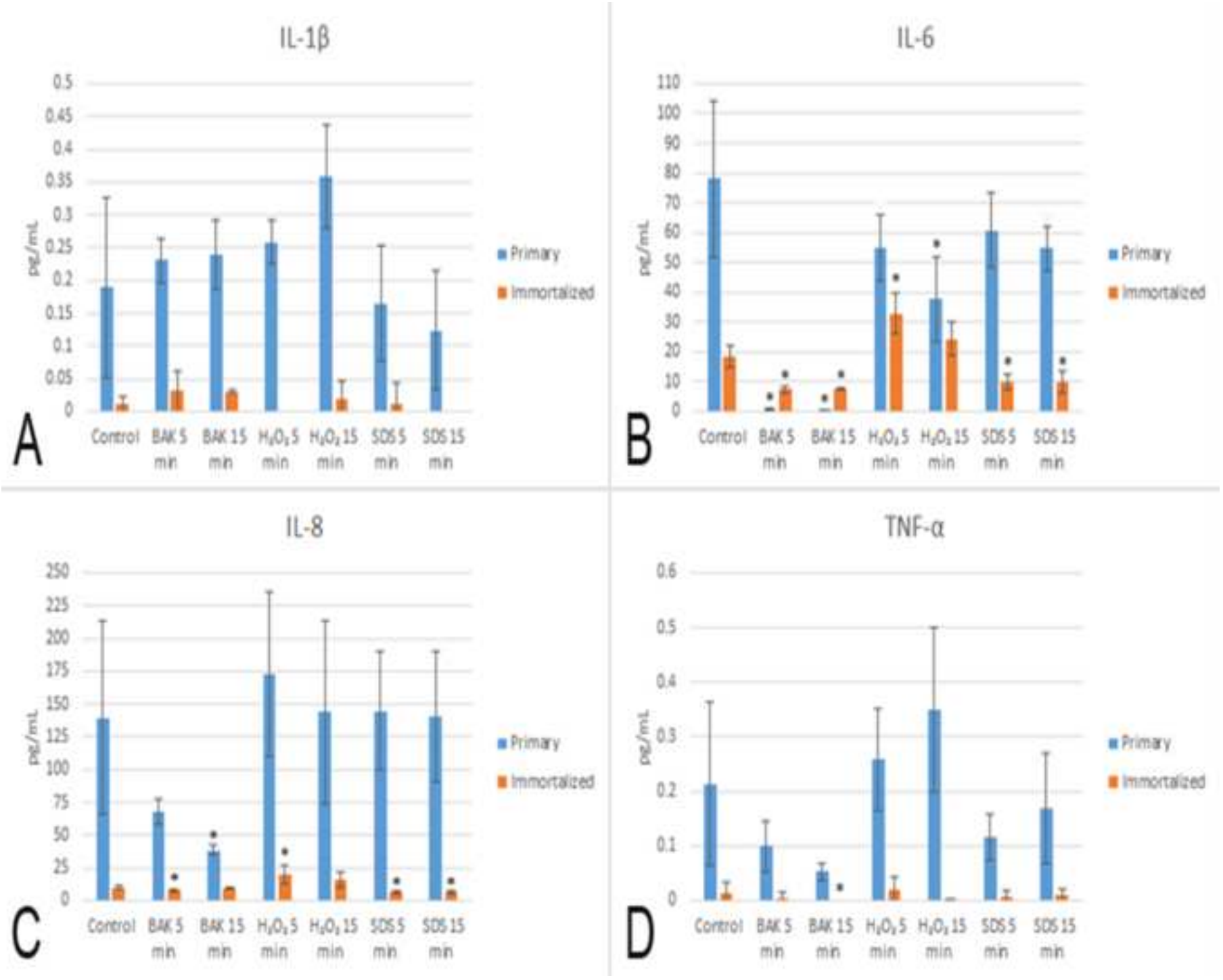
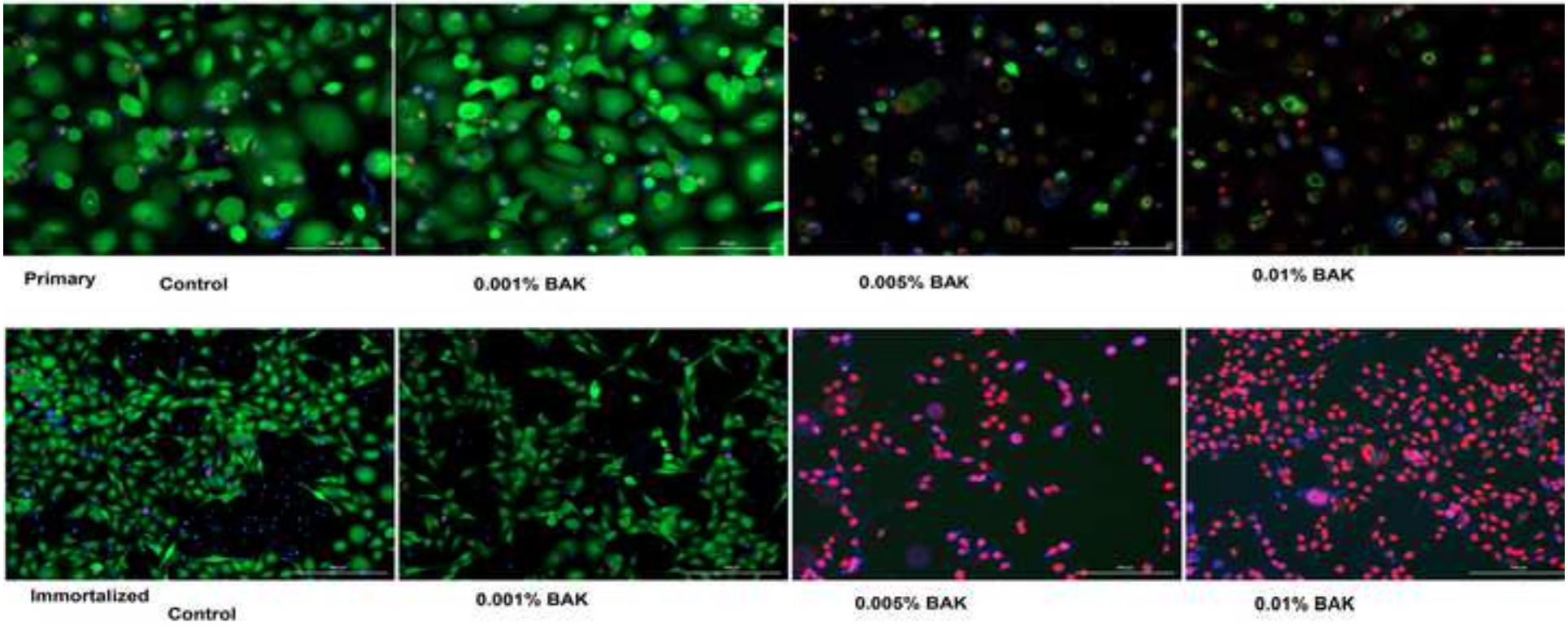


Figure 7



CORE Centre for Ocular Research & Education

Vidhya Lyer, Ph.D. Review Editor
Journal of Visualized Experiments (JoVE)
1 Alewife Center, Suite 200
Cambridge, MA 02140

Subject: Submission of revised manuscript

Revision Submission Date: July 5, 2021

Dear Dr. Lyer,

Thank you for reviewing our manuscript "Methods for Determining the Toxicity of UV Radiation and Chemicals on a Primary and an Immortalized Human Corneal Epithelial Cell Line JoVE 62675". Please find below our comments and changes described below on the review of our manuscript.

Editorial comments:

I have included the comments and response in this response memo. These same responses to the comments are also included in the comments of the revised manuscript.

Comment 1. This seems to be a proprietary name for something that's either EGF or a mixture of such growth factors. Please consider replacing epiFactor O and epiFactor P with generic terms that can be inserted in the comment's column next to those items in the Table of Materials.

Response 1. Replaced epiFactor with the generic term cell media supplement and put epifactor O and P into the comments section of the Table of Materials

Comment 2. All three for all three lasers or any particular order?

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Response 2. Clarified the use of the HFT NFT and LP beam splitters used with the lasers for obtaining a confocal image.

Comment 3. Is this both radiation intensities for both times or particular combinations?

Response 3. Clarified that the UVA and UVB lamps are turned on at the same time. This combined radiation then is used to expose the cells at 5 minutes and then for a separate experiment with different cells at 20 minutes.

Comment 4. Original Comment. Collect it for some reason or aspirate? Can this be deleted as you mention the same thing in the next step?

Response 4. Rearranged the text in section 3 to avoid duplication.

Comment 5. Please combine both panels A and B into one figure to fit into one page.

Response 5. Figure 2 panels A and B have been combined.

Comment 6. For Figure 3 What is the y-axis label mean "Percent No. UV control"? Percent relative to non-UV exposed control?

Response 6. Clarified in figure caption what the y axis label means.

Comment 7. For Figure 5 What is the y-axis label mean "Percent No. Toxin control"? Percent relative to untreated control?

Response 7. Clarified in figure caption what the y axis label means.

Comment 8. For figure 6 Please replace "mins" with "min" everywhere in your figure.

Response 8. Replaced all mins with min in this figure.

Comment 9. Please connect SV40-immortalized cells with your iHCECs so readers can understand the relevance.

Response 9. Clarified that the immortalized cell line used in this investigation was an SV40 immortalized cell line.

Comment 10. Original comment Here, it seems you are making the case for the inhibition of Rb, p53, and pp2a to make cells progress through the cell cycle unimpeded. Please replace "interfere" with "promote cell cycle progression".

Response 10. Interfere was replaced with promote cell cycle progression.

Comment 11. As well as CORE or through CORE?

Response 11. Clarified that the research support was through CORE.

DISCLOSURES:
The author, Lyndon W. Jones, over the past 3 years, through CORE has received research support or lectureship honoraria from the following companies: Alcon, Allergan, Allied Innovations, Aurinia Pharma, BHVI, CooperVision, GL Chemtec,i-Med Pharma, J&J Vision, Lubris, Menicon, Nature's Way, Novartis, Ophtecs, Ote Pharma, PS Therapy, Santen, Shire, SightGlass SightSage, and Visioneering. Lyndon Jones is also a consultant and/or serves on an advisory board for Alcon, CooperVision, J&J Vision, Novartis, and Ophtecs. The other authors have nothing to disclose.

Sincerely,

David McCanna

Corresponding Author

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